Photoöxidation of Crystalline Lysozyme in the Presence of Methylene Blue and Its Relation to Enzymatic Activity

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Introduction

The effect of visible light in the presence of methylene blue on amino acids and proteins has been investigated in our previous work (1, 2). The study on crystalline lysozyme reported here was an extension of that work and of our preliminary work on lysozyme (3). It was undertaken to correlate the observed chemical and physical changes brought about by photoöxidation with the enzymatic activity.

Experimental

Crystalline lysozyme purchased from the Armour laboratories[2] was used for the irradiation work. The manometric technique for the irradiation was described in our previous papers (4, 5). Calculation of the lysozyme concentration was based on the nitrogen content of the enzyme as 18.6% (6, 7).

Effect of pH on the Photochemical Action of Methylene Blue on Lysozyme

One ml. of solution containing 5 mg. lysozyme (adjusted to pH values of 4.5, 5.8, 6.8, 7.8, 8.7, and 9.2) and 0.5 ml. of 0.2 *M* phosphate buffer of the appropriate pH were put in the main chamber of the Warburg vessel. In the side arm of the vessel, 0.5 ml. of aqueous methylene blue solution containing 0.1 mg. dye was placed. Because of the accompanying CO_2 evolution during photoöxidation, concentrated KOH was placed in the center well of the vessel in all the experiments. After temperature equilibration at 37°, the contents of the vessel were mixed, and irradiation was started. The results indicated that the photoöxidation of lysozyme increased with increased pH values, reaching a maximum at about pH 9.0.

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[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] Mention of a product does not imply that it is endorsed or recommended by the U. S. Department of Agriculture over similar products not mentioned.

Photochemical Action of Methylene Blue on the Histidine, Tryptophan, Tyrosine, Cystine and Methionine Contents of Lysozyme and on Its Enzymatic Activity

In view of our previous findings (1, 2), which showed a high degree of susceptibility of histidine, tryptophan, tyrosine, cystine, and methionine to photoöxidation, the rate of oxidation of these amino acids within the lysozyme molecule was studied at various levels of oxygen uptake. The analytical methods used for determination of these amino acids were those described previously (2). Calculation of the moles of oxygen uptake was based on the molecular weight of lysozyme as 15,000 (6, 7, 8). For determination of the amino acids at a given level of oxygen uptake, 1 ml. of solution containing 10 mg. of lysozyme adjusted to pH 8.7, 0.5 ml. of 0.2 *M* phosphate buffer of pH 8.7, and 0.5 ml. of 0.02% methylene blue solution were mixed and irradiated for the desired period. Determination of lysozyme activity was based on the method of Boasson (9). A killed culture of *Micrococcus lysodeikticus* was suspended in a 0.85% solution of NaCl so that the light transmission in a 1-cm. cell was 20%; a No. 425 filter was used in the Fisher electrophotometer. To 1 ml. of this bacterial suspension were added 1 ml. of a 0.2 *M* phosphate buffer of pH 6.2 and a 1-ml. aliquot of a solution containing 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 μg. lysozyme. Turbidity was measured after 15 min. incubation at 37°. The values, obtained in triplicate, did not vary more than 5%. Under these conditions, the logarithm of the observed light transmission was proportional to the enzyme concentration, and thus could be used with fair accuracy for the quantitative measurement of lysozyme activity. Variations in the calibration curve were observed from culture to culture, however, and it is, therefore, essential to set up a new calibration whenever a new bacterial suspension is used.

The effect of photoöxidation on the lysozyme activity was observed in experiments carried out as described for the amino acid determination; only 5 mg. of lysozyme was used. At the desired degree of oxygen uptake, the contents of the vessel were diluted so that it contained 1 μg. lysozyme/ml., and the lysozyme activity in 1 ml. was determined in triplicate, as described before. The irradiation times for 0.52, 1.50, 2.20, 3.7, 6.0, 11.2, and 23.3 moles O_2 uptake/mole lysozyme were: 3, 7, 9, 11, 25, 43, and 142 min., respectively. Control experiments showed that, if the irradiation of the enzyme was carried out over these periods without the dye, the enzymatic activity was not altered. Simi-

lar experiments carried out in the presence of methylene blue in a nitrogen atmosphere showed no decrease in the original enzyme activity, thus demonstrating that the observed enzyme inactivation was entirely due to photoöxidation.

Figure 1 shows the changes produced in amino acid composition and activity of lysozyme by photoöxidation. The total histidine (1 mole) content of the enzyme was rapidly photoöxidized, and reached zero at 2 moles O_2 uptake/mole enzyme. At this point, 17% of the total tryptophan content and 70% of the enzymic activity were gone, but no changes in tyrosine, methionine, and cystine contents could be ob-

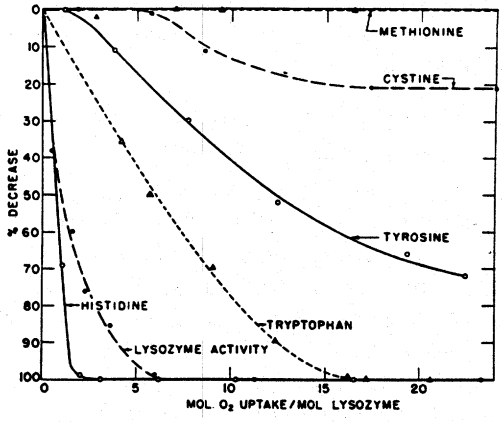

FIG. 1. Decrease of various amino acids and of enzymatic activity during the photochemical action of methylene blue on lysozyme.

served. At 6 moles O_2 uptake/mole enzyme, the entire enzymic activity was eliminated, and a 50% decrease in tryptophan content and a 19% decrease in the tyrosine content had taken place. The methionine and cystine contents were still not altered at this point. Further photooxidation produced a progressive decrease of tryptophan, tyrosine, and cystine of varying degrees, whereas the methionine content was not changed at all, as was observed previously for β-lactoglobulin (2).

Effect of the Photochemical Action of Methylene Blue on the Ultraviolet Absorption Spectrum of Lysozyme

Lysozyme was irradiated under the same conditions used for the experiments shown in Fig. 1, and the absorption spectrum was measured

in a solution of appropriate dilution, as described in our previous paper (2). The results show that with progressive photoöxidation of lysozyme the extinction coefficient ($E^{1\%}_{1cm.}$) of the absorption maximum at 280 mμ was reduced from 28.7 to 20.0 if the enzyme were photoöxidized to 5.3 moles of oxygen uptake. At 12.5 and 23.4 moles of O_2 uptake/mole enzyme, further reduction of the extinction coefficient at 280 mμ took place (16.2 resp. 12.0), and the peak in the curve disappeared. The absorption minimum of the original enzyme at 252 mμ was shifted to 255 mμ at 3.5 moles O_2 uptake/mole enzyme, and the $E^{1\%}_{1cm.}$ values at these points dropped from 22.5 to 18.7. At 5.3 moles of oxygen uptake the minimum shifted to 258 mμ and the observed extinction coefficient was 18.5. At 12.5 and 23.4 moles of oxygen uptake, no absorption minimum could be observed.

Effect of the Photochemical Action of Methylene Blue on the Solubility and Viscosity of Lysozyme

In the solubility studies, the lysozyme preparation was dissolved in 0.1 *N* NaCl solution and adjusted with dilute NaOH solution to pH 10.7, the isoelectric point of the enzyme. To 1.5 ml. of this solution containing 10 mg. of the enzyme was added 0.5 ml. of 0.1 *N* NaCl solution containing 0.1 mg. of methylene blue, and the mixture was irradiated as described previously. With oxidation a precipitate was formed, which increased with progressive oxygen uptake. At given degrees of photoöxidation, the contents of the Warburg vessel were transferred and centrifuged, and the total nitrogen content of the supernatant was determined to calculate the enzyme content remaining in solution. Resuspension of the precipitate in 2 ml. of 0.1 *N* NaCl solution and successive centrifugation resulted in supernatants free of protein (determined by total nitrogen determinations), and thus the observed decrease in solubility of lysozyme was not obscured by partial solubility in the medium. Control experiments carried out in the absence of the dye but irradiated for the same time (120 min.) showed no precipitate, nor did we find any decrease in enzymatic activity under these conditions. The observed stability of lysozyme at pH 10.7 is in agreement with the results of earlier work of Alderton, Fevold, and Lightbody (10).

The technique for measuring the changes in the relative viscosity of lysozyme during photoöxidation was identical with that described for β-lactoglobulin (2). The lysozyme solution was adjusted to pH 7.4, and

to 1.5 ml. of the solution containing 30 mg. of enzyme was added 0.5 ml. of solution containing 0.1 mg. of dye. The mixture was irradiated as previously described. At pH 7.4, the photoöxidation did not produce a visible precipitate and did not complicate the viscometric measurement. The relative viscosity was measured at various stages of photooxidation. Control experiments without the dye showed no changes in viscosity during the irradiation. Results of the solubility and viscosity studies (Fig. 2) showed a progressive decrease in solubility and an increase in viscosity of the enzyme during photoöxidation.

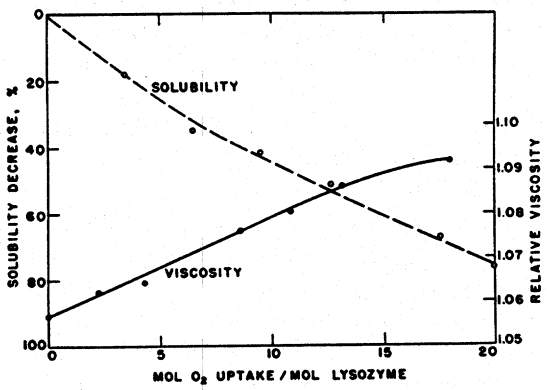

FIG. 2. Changes in solubility and viscosity of lysozyme produced by the photochemical action of methylene blue.

Does the Photoinactivated Lysozyme Exert an Inhibiting Action on the Native Enzyme?

Lysozyme was photoöxidized as described for the experiments presented in Fig. 1 until 6 moles of oxygen was taken up per mole of enzyme, at which point, as shown in Fig. 1, the activity of the enzyme was completely eliminated. To 1 μg. of native enzyme were added aliquots containing 0.4, 0.8, 1.2, and 1.6 μg. of photoinactivated enzyme, respectively, and the activity was measured as described before. The result gave no indication of inhibition by the irradiated enzyme.

DISCUSSION

The hydrolytic enzymes isolated so far in crystalline form proved to be protein, and, in contrast to the crystalline respiratory enzymes, no

prosthetic groups could be demonstrated. The catalytic properties of the hydrolytic enzymes, therefore, might be ascribed to a particular configuration of amino acids in the protein molecule itself. This assumption is supported by studies on the chemical modification of various enzymes (11, 12, 13), which have demonstrated the possible importance of certain groupings within the enzyme molecule to activity.

In view of these findings, we extended our studies on the photochemical action of methylene blue to lysozyme, with the hope that the changes brought about in this manner might be correlated with the enzymatic activity of lysozyme. The method offers the advantages of a certain degree of selectivity, mild conditions, and the absence of strong chemicals, except the traces of dye, which can be easily removed if desired.

Our results showed that, just as in the case of β-lactoglobulin (2), the histidine and tryptophan molecules of the enzyme are first affected by the photochemical action of methylene blue. At 2 moles O_2 uptake/mole enzyme, the total histidine content (1 mole) and 1.2 moles tryptophan of the total of 8 moles were photoöxidized, whereas the enzymatic activity decreased by 70%. At this point, no changes in tyrosine, cystine, and methionine content could be observed. At 6 moles O_2 uptake/mole enzyme, the enzymatic activity was completely eliminated, and at this point a decrease of 4 moles tryptophan of the total of 8 moles and a decrease of 0.57 mole tyrosine of the total of 3 moles were observed; the cystine and methionine contents were not affected.

It appears that lysozyme retains about 30% of its activity, even after complete elimination of histidine, and this residual activity is eliminated at a slower rate only after the photoöxidation of three additional molecules of tryptophan. The remaining four units of tryptophan apparently are not involved in the catalytic action of lysozyme. The possible participation of tyrosine in the enzymatic activity is unlikely, since at complete inactivation of the enzyme only about one-half a molecule was photoöxidized, instead of a whole unit, as would be required. This assumption is in partial agreement with Fraenkel-Conrat (14), who ascribes to this group only a minor role.

The fact that the rate of decrease in histidine follows closely the rate of enzyme inactivation (up to about 70%) indicates the possible participation of this amino acid in the enzymatic activity of lysozyme. The work of Fraenkel-Conrat (14) also indicates that this might well be the case. In addition, Fraenkel-Conrat claims that the chemical

modification of the amino, carboxyl, amide, guanidine, and hydroxyl groups of lysozyme has a detrimental effect on the enzyme activity, whereas modification of the indole group is less harmful. This latter finding appears to be in disagreement with our previous (3) and present findings, and also with the recent paper of Shugar (15).

Progressive photooxidation of lysozyme produces gradual denaturation and an increase in viscosity of the molecule. These physical changes do not appear to be related directly to the decrease in enzymatic activity, inasmuch as a 70% inactivated enzyme was denatured to only 11%, whereas at 100% inactivation the denaturation did not exceed 27%.

Our results indicate that histidine might represent a contributing factor in lysozyme activity, together with half the tryptophan present in the lysozyme molecule (8 moles). The possible role of other groups in the enzyme which might act in conjunction with these groups awaits further investigation.

Summary

Optimal conditions for the photooxidation of lysozyme were investigated.

Photooxidation of the lysozyme molecule to the extent of 2 moles of oxygen resulted in 70% reduction in enzymatic activity, in complete elimination of histidine (1 mole), and in loss of 1.2 moles tryptophan out of the total of 8 moles.

Photooxidation carried out to 6 moles of oxygen per mole of enzyme eliminated the activity completely, and caused an additional loss of about 3 moles of tryptophan and about a half mole of tyrosine of the total of 3 moles.

Changes in the ultraviolet absorption spectrum of lysozyme during photooxidation are presented.

The observed decrease in solubility and increase in viscosity during photooxidation of lysozyme did not appear to be related to enzyme inactivation.

The possible roles of tryptophan and histidine in lysozyme activity are discussed.

References

1. Weil, L., Gordon, W. G., and Buchert, A. R., *Arch. Biochem. Biophys.* **33,** 90 (1951).
2. Weil, L., and Buchert, A. R., *Arch. Biochem. Biophys.* **34,** 1 (1951).

3. WEIL, L., AND MAHER, J., *Federation Proc.* **9,** 244 (1950).
4. WEIL, L., *Science* **107,** 426 (1948).
5. WEIL, L., AND MAHER, J., *Arch. Biochem.* **29,** 241 (1950).
6. LEWIS, J. C., SNELL, N. S., HIRSCHMANN, D. J., AND FRAENKEL-CONRAT, H., *J. Biol. Chem.* **186,** 23 (1950).
7. FROMAGEOT, C., AND DE GARILHE, M. P., *Biochim. et Biophys. Acta* **4,** 509 (1950).
8. FEVOLD, H. L., *Advances in Protein Chem.* **6,** 230 (1951).
9. BOASSON, E. H., *J. Immunol.* **34,** 281 (1938).
10. ALDERTON, G., FEVOLD, H. L., AND LIGHTBODY, H. D., *Federation Proc.* **5,** 119 (1946).
11. HERRIOTT, R. M., AND NORTHROP, J. H., *J. Gen. Physiol.* **18,** 35 (1934).
12. SIZER, I. W., *J. Biol. Chem.* **160,** 547 (1945).
13. JANSEN, E. F., NUTTING, M. D. F., JANG, R., AND BALLS, A. K., *J. Biol. Chem.* **179,** 189 (1949).
14. FRAENKEL-CONRAT, H., *Arch. Biochem.* **27,** 109 (1950).
15. SHUGAR, D., *Bull. soc. chim. biol.* **33,** 710 (1951).